https://doi.org/10.1590/1678-4499.20230099

PLANT BREEDING
Article

Rpp genes conferring resistance to Asian soybean rust in F_2 population in the field conditions

Daniela Meira[1], Vinícius de Bitencourt Bez Batti[1], Leomar Guilherme Woyann[1], Anderson Simionato Milioli[1], Antonio Henrique Bozi[1], Eduardo Beche[2], Maiara Cecilia Panho[1], Fabiana Barrinouevo[1], Laura Alexandra Madella[1], Gaspar Malone[3], Salvador Lima Brito Júnior[3], Taciane Finatto[1], Giovani Benin[1,*]

1. Universidade Tecnológica Federal do Paraná – Agronomia - Pato Branco (PR), Brazil.
2. University of Missouri – Division of Plant Science, Columbia, Missouri, MO 65211, USA.
3. GDM Seeds – Londrina (PR), Brazil.

Received: Jan. 27, 2023 | **Accepted:** Sep. 4, 2023

Section Editor: Carlos Alberto Scapim

***Corresponding author:** benin@utfpr.edu.br

How to cite: Meira, D., Batti, V. B. B., Woyann, L. G., Milioli, A. S., Bozi, A. H., Beche, E., Panho, M. C., Barrinouevo, F., Madella, L. A., Malone, G., Brito Júnior, S. L., Finatto, T. and Benin, G. (2023). *Rpp* genes conferring resistance to Asian soybean rust in F_2 population in the field conditions. Bragantia, 82, e20230099. https://doi.org/10.1590/1678-4499.20230099

ABSTRACT: In this study, the aim of this study was to identify the source of resistance using KASP markers developed for *Rpp1* – *Rpp5* and screening for resistance in field trials in F_2 populations. Ten F_2 soybean (*Glycine max* (L.) Merrill) populations derived from crosses between rust-susceptible (55I57RSF IPRO, 63I64RSF IPRO) and rust-resistant sources (PI 200492, PI 594538A, PI 587880A, PI 594723, PI 230970, PI 506764, PI 459025A and PI 200487) were evaluated. All F2 plants were individually evaluated in field conditions for ASR phenotypic reactions and classified according to sporulation level. KASP markers were developed according to assays associated with *Rpp* genes available at SoyBase. Based on a slight difference in map position and different phenotypic disease reactions of PI 200492, we suggest that PI 594723 carries a resistance gene *Rpp1-b*. The *Rpp1-b* gene from PI 594723 was mapped on Chr 18 in a 12.4 cM region. The PIs carrying *Rpp1-b* (PI 594723, PI 587880A, and 594538A) showed strong resistance to ASR compared to the lines carrying *Rpp1* (PI 200492). A total of 26 KASP markers were significantly associated ($P < 0.01$) with ASR resistance. Among those, M1, M5, and M6 (*Rpp1*), M13 and M14 (*Rpp2*), M16, M17 and M20 (*Rpp3*), M25 and M26 (*Rpp4*), and M27 and M28 (*Rpp5*) have the potential to be used in marker-assisted selection strategies.

Key words: *Phakopsora pachyrhizi*, linkage mapping, KASP markers, *Glycine max*.

INTRODUCTION

Asian soybean rust (ASR), caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd, is considered as one of the most damaging soybean (*Glycine max* (L.) Merrill) diseases worldwide (Godoy et al. 2016; Langenbach et al. 2016). Because of the pathogen dissemination and yield losses, greater effort is needed to discover new resistant sources and genes (Meira et al. 2020). The pathogen can infect 31 leguminous species in natural conditions, such as *G. max*, *Glycine soja*, and *Vigna unguiculata*, and more than 60 different species in controlled conditions (Goellner et al. 2010).

Phakopsora pachyrhizi is present in the main soybean producer regions, mainly because of the windborne urediniospores dispersion. When the urediniospores reach the leaf, ideal conditions of surface moisture and temperature (17 to 28 °C) initiate the germination process in a couple of hours. Most rust species enter the leaf through the stomata, but *P. pachyrhizi* utilizes an appressorial peg to directly penetrates the leaf epidermal cell wall. After the latent period of five to eight days, small chlorotic spots on older leaves are observed on the abaxial side. Later the lesions advance to volcano-shaped uredina, which produce innumerous urediniospores responsible for the new infection cycle (Goellner et al. 2010). ASR causes early defoliation and reduces photosynthetic area, resulting in yield losses and increased costs (Godoy et al. 2016; Langenbach et al. 2016).

Several methods have been developed to control ASR, such as field monitoring, elimination of secondary hosts, use of soybean-free periods to break the fungus cycle, fungicides, and genetic resistance (Kendrick et al. 2011). In the last few years, fungicide efficiency has decreased due to the intense use and lower pathogen sensitivity to different fungicide modes of action (Goellner et al. 2010; Langenbach et al. 2016). Thus, soybean breeders and geneticists have focused on incorporating genetic resistance or tolerance in high-yielding materials, combining different control methods through an integrated management approach.

The *Rpp* genes confer hypersensitivity reactions to the pathogen known as *reddish brown* (RB) lesions (Miles et al. 2011). The resistant phenotype to ASR is classified in RB1 to RB3 according to the level of sporulation, in contrast the susceptible phenotype presents abundant sporulation, known as TAN lesions. The hypersensitive response occurs when the plant detects microbial effectors by plant resistance proteins (R), elicits effector-triggered immunity, and promotes localized cell death (Boller and Felix 2009). Until now, seven different loci of qualitative resistance have been identified: *Rpp1* on the chromosome (Chr) 18 (Hyten et al. 2007); *Rpp2* on Chr 16 (Silva et al. 2008; Yu et al. 2015); *Rpp3* on Chr 6 (Hyten et al. 2009); *Rpp4* on Chr 18 (Garcia et al. 2008; Hartwig 1986); *Rpp5* on Chr 3 (Garcia et al. 2008); *Rpp6* on Chr 18 (Li et al. 2012); and recently *Rpp7* on Chr 19 (Childs et al. 2018), and two different alleles of *Rpp1* were described, *Rpp1-b* (Chakraborty et al. 2009; Chen et al. 2015) and *Rpp1*? (Ray et al. 2009).

Long-term resistance is difficult to achieve due to the diversity of pathogen isolates and race-specific monogenic resistance of each *Rpp* gene against ASR isolates (Aoyagi et al. 2020). The same resistance source may present different phenotypic reactions according to the geographic origin of the isolate. In general, the Brazilian ASR isolates are known as the most aggressive (Aoyagi et al. 2020). The *Rpp1* (PI 200492) is highly resistant to Japanese and Mexican ASR isolates, but a lack of resistance has been reported to Brazilian ASR isolates (Akamatsu et al. 2017; Aoyagi et al. 2020; Hossain et al. 2015). In contrast, the *Rpp1-b* (PI 587880A, PI 594538A) is resistant to most Brazilian ASR isolates (Akamatsu et al. 2017; Ray et al. 2009; Yamanaka et al. 2016, including field isolates (Panho et al. 2022), highlighting a clear allelic difference between *Rpp1* and *Rpp1-b*.

Introgression of resistance genes from plant introductions (PIs) in elite lines is an efficient way to develop varieties resistant to ASR. Marker-assisted selection (MAS) may enable selection in early generations, reducing phenotyping time and selecting only plants with the desirable allele combination, and identifying genes. Single nucleotide polymorphism (SNP) markers have been used extensively, mainly due to high throughput and low cost. In this way, a genotyping strategy using KASP (Kompetitive Allele-Specific PCR) methodology is a high-throughput and breeder-friendly.

The hypothetically *Rpp1-b* presents in PI 594723 due to the phenotype similarity to PIs carrying *Rpp1-b* (Li 2009) can be verified using MAS and KASP markers. Thus, the aim of this study was to identify the source of resistance using KASP markers developed for *Rpp1* – *Rpp5* and screening for resistance in field trials in F_2 populations.

METHODS

Plant material

Ten F2 populations derived from single crosses between different soybean rust-susceptible cultivars and the resistant sources (PIs) carrying *Rpp* genes were made (Table 1). The susceptible parents (55I57RSF IPRO and 63I64RSF IPRO) used in the crosses were highly cultivated in Brazil. Crosses were performed in two years, in 2017 for populations 1 to 4 and 2018 for populations 5 to 10. The resistant sources (PIs) were used as males, and the susceptible soybean cultivars were female. F_1 hybrids were grown in greenhouse conditions, and seeds were bulk harvested to produce the F_2 generation.

Crosses were performed in a greenhouse at GDM Genética do Brasil, in Porto Nacional, State of Tocantins - Brazil. The F1s obtained were advanced in greenhouse. The field experiments were designed to evaluate all the F_2 populations and were performed in two phases in the experimental area of GDM Genética do Brasil, in Cambé, State of Paraná - Brazil. First, during the 2017/18 crop season, F_2 plants of populations 1 – *Rpp1* (54 individuals), 2 – *Rpp1-b* (75 individuals), 3 – *Rpp1-b* (70 individuals), and 4 – *Rpp1** (69 individuals) were grown. During the 2018/19 crop season, F_2 plants of populations 5 – *Rpp1** (298 individuals), 6 – *Rpp2* (277 individuals), 7 – *Rpp3*, 5 (288 individuals), 8 – *Rpp4* (291 individuals), 9 – *Rpp5* (284 individuals), and 10 – *Rpp5* (284 individuals) were grown (Table 2).

Table 1. Crosses between the soybean rust-susceptible parental and resistant sources (PI) and crop season of field experiments.

Population	Crop Season	Susceptible parental	Resistance source (PI number) – Rpp	Chr	Origin[9]
1 – *Rpp1*	2017/18	55I57RSF IPRO	Komata (PI 200492) – *Rpp1*[1]	18	Japan
2 – *Rpp1-b*	2017/18	55I57RSF IPRO	(PI 594538A) – *Rpp1-b*[2]	18	China
3 – *Rpp1-b*	2017/18	55I57RSF IPRO	Huang Dou (PI 587880A) – *Rpp1-b*[3]	18	China
4 – *Rpp1**	2017/18	55I57RSF IPRO	He xian hei dou (PI 594723) - *Rpp1**[4]	18	China
5 – *Rpp1**	2018/19	63I64RSF IPRO	He xian hei dou (PI 594723) - *Rpp1**[4]	18	China
6 – *Rpp2*	2018/19	55I57RSF IPRO	PI 230970 - *Rpp2*[5]	16	Japan
7 – *Rpp3, 5*	2018/19	55I57RSF IPRO	Hyuuga (PI 506764) - *Rpp3, Rpp5*[6]	6, 3	Japan
8 – *Rpp4*	2018/19	55I57RSF IPRO	Bing nan (PI 459025A) - *Rpp4*[7]	18	China
9 – *Rpp5*	2018/19	55I57RSF IPRO	Kinoshita (PI 200487) - *Rpp5*[8]	3	Japan
10 – *Rpp5*	2018/19	63I64RSF IPRO	Kinoshita (PI 200487) - *Rpp5*[8]	3	Japan

[1] PI 200492 – Hyten et al. (2007). [2] PI 594538A - Chakraborty et al. (2009). [3] PI 587880A- Ray et al. (2009). [4]PI 594723 – Miles et al. (2006); [5]PI 230970 – Hartwig and Bromfield (1983); [6]PI 506764 – Kendrick et al. (2011); [7] PI 459025A – Hartwig (1986); [8]PI 200487 – Garcia et al. (2008); [9]GRIN - Germplasm Resources Information Network. Natl. Germplasm Resource. https://www.ars-grin.gov/ *PI 594723 carrying an *Rpp1* allele not mapped until this moment. Source: Elaborated by the authors using data from Hyten et al. (2007), Chakraborty et al. (2009), Ray et al. (2009), Miles et al. (2006), Hartwig and Bromfield (1983), Kendrick et al. (2011), Hartwig (1986), Garcia et al. (2008), 9GRIN - Germplasm Resources Information Network. Natl. Germplasm Resource. https://www.ars-grin.gov/.

Table 2. Segregation ratios of phenotypic reaction to Asian soybean rust (ASR) in F_2 soybean populations.

Pop - *Rpp* gene	Crossing	Number of plants			χ^2 R:S (3:1)	χ^2 p(<0.05)
		R	S	Total		
P1 - *Rpp1*	55I57RSF IPRO x PI 200492	27	27	54	6.75	0.00
P2 - *Rpp1-b*	55I57RSF IPRO x PI 594538A	59	16	75	0.20	0.46
P3 - *Rpp1-b*	55I57RSF IPRO x PI 587880A	55	15	70	0.18	0.49
P4 - *Rpp1**	55I57RSF IPRO x PI 594723	54	15	69	0.15	0.53
P5 - *Rpp1**	63I64RSF IPRO x PI 594723	210	88	298	3.26	0.07
P6 - *Rpp2*	55I57RSF IPRO x PI 230970	172	105	277	24.61	0.00
P7 - *Rpp3,5*	55I57RSF IPRO x PI 506764	213	75	288	10.05[1]	0.00
P8 - *Rpp4*	55I57RSF IPRO x PI 459025A	261	30	291	69.42	0.00
P9 - *Rpp5*	55I57RSF IPRO x PI 200487	226	58	284	3.17	0.07
P10 - *Rpp5*	63I64RSF IPRO x PI 200487	230	54	284	5.43	0.02

R: resistant plants; S: susceptible plants. [1]Segregation expected 13:3. Source: Elaborated by the authors.

The resistance sources and susceptible cultivars had four replications in each crop season. Each plot was composed of two rows spacing of 0.5 m and a row length of 3 m, with a density of 10 seed·m^{-1}. An experimental planter was used for sowing the populations on a non-preferential date (December) to enable the natural occurrence and development of Asian soybean rust. No fungicide application was made to control the disease.

Resistance evaluation

All F_2 plants were evaluated individually, totaling 1990 individuals used to disease rating, and 50 plants from each resistant and susceptible parent were rated. All F2 plants were evaluated for ASR phenotypic reactions in the R5 growth stage (Fehr et al. 1971). Three infected leaves in the middle third of each plant were visually evaluated, according to a sporulation level (SL) scale adapted from Yamanaka et al. (2010) and Miles et al. (2011). Lesion types were recorded as immune (IM - 0), no sporulation of *reddish-brown* lesions (RB1 - 1), little sporulation (RB2 - 2), moderate sporulation (RB3 - 3), and reaction for abundant sporulation (TAN - 4) (Fig. 1).

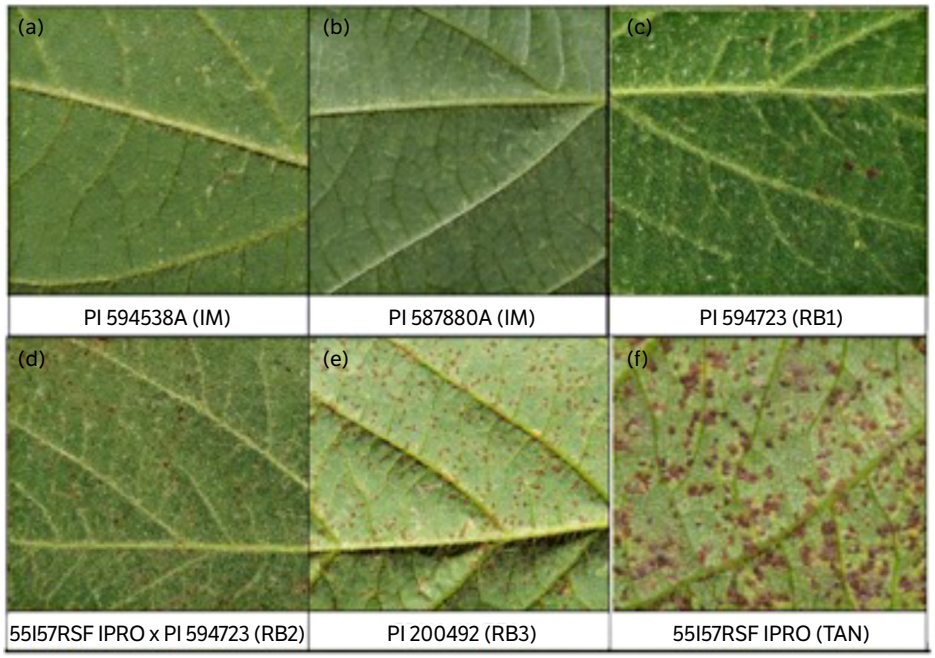

Figure 1. Evaluation scale used for rating the phenotypic reaction to *Phakopsora pachyrhizi* in soybean genotypes and F2 populations, classified as immune (IM - 0), no sporulation of reddish-brown lesions (RB1 - 1), little sporulation (RB2 - 2), moderate sporulation (RB3 - 3), and reaction for abundant sporulation (TAN - 4). a) PI 594538A (*Rpp1*-b), b) PI 587880A (*Rpp1*-b), c) PI 594723 (*Rpp1*), d) 55i57RSF IPRO x PI 594723, e) PI 200492 (*Rpp1*), and f) 55i57RSF IPRO.

Source: Elaborated by the authors.

KASP markers

The SNP markers used in this study were developed based on molecular markers linked to *Rpp* genes available at the SoyBase (https://www.soybase.org – Wm82 Glyma 2.0) and in the literature (Table 3). In the first step, the susceptible and resistant parents were analysed by genotyping by sequencing to explore in high density the SNPs around the mapped gene region. Using this information, polymorphic markers between parents were defined to use in gene mapping and marker assisted selection. Twelve KASP markers were used to map the *Rpp1* gene in populations 1 to 4 (Supplementals Table S1, Table S2). KASP markers highly associated with *Rpp1* (Supplemental Table S3) and markers developed for *Rpp2*, *Rpp3*, *Rpp4*, and *Rpp5* were used to map *Rpp* genes in populations 5 to 10 (Supplemental Table S2).

Table 3. Resistance loci to Asian soybean rust (ASR) mapped with molecular markers available at the SoyBase (https://www.soybase.org).

PI	Rpp	Chr	Marker start – end (physical position)		Distance (kb)	Reference
PI 200492	*Rpp1*	18	Sat_187 (56181759)	Sat_064 (56333703)	152.1	Hyten et al. 2007
PI 594538A	*Rpp1-b*	18	Sat_064 (56333703)	Sat_372 (56333845)	463.5	Chakraborty et al. 2009
PI 587880A	*Rpp1-b*	18	Sat_064 (56333703)	Satt191 (54450956)	1882.1	Ray et al. 2009
PI 594723	*Rpp1**	18	-	-	-	-
PI 230970	*Rpp2*	16	BARCSOYSSR_16_0902 (29253155)	BARCSOYSSR_16_0908 (26441233)	188.1	Yu et al. 2015
PI 506764	*Rpp3,*	6	satt460 (44049891),	Satt307 (46820834),	2770.9	Monteros et al. 2007;
	Rpp5	3	Satt275 (29862641)	Sat_280 (32670690)	2808.1	Kendrick et al. 2011
PI 459025A	*Rpp4*	18	Satt288 (51127425)	Af162283 (57436765)	6309.4	Silva et al. 2008
PI 200487	*Rpp5*	3	Satt275 (29862641)	Sat_280 (32670690)	2808.1	Lemos et al. 2011

Source: Elaborated by the authors using the references cited.

DNA analysis

DNA was extracted from young leaf tissue of each F2 plant at the V4 growth stage, using a silica column kit of LGC Genomics (Teddington, UK). Genotyping assays were tested in a 96-well format and set up as 10 µL reactions (4.85 µL of template (50–75 ng of DNA), 5.0 µL of 2 x Kaspar mix, and 0.15 µL of primer mix). PCR was performed according to the protocol: an initial 15 min at 94 ºC; 10 *Touchdown* cycles of 94 ºC for 20 s, 65-57 ºC for 60 s (dropping 0.8 ºC per cycle); 26 amplification cycles of 94 ºC for 20 s, 57 ºC for 60 s; with final extension for 7 min at 72 ºC. The fluorescence data were collected in the pre-read and post-read stages (37 ºC for 1 min). Data were automatically processed using KBioscience Kraken software and visually checked using KBioscience SNPViewer (LGC Limited, UK).

Statistical analysis

Observed and expected segregation ratios of ASR resistance and KASP markers were tested using Chi-square (χ^2) analysis. The expected segregations were 1:2:1 (dominant homozygous, heterozygous, and recessive homozygous) to markers, 3:1 (resistance and susceptibility to ASR) to phenotype, and 15:1 to population 7 (*Rpp3*, 5) with two genes. Phenotypic data were converted into resistant (R) summing IM - 0, RB1 - 1, and RB2 - 2 plants; and susceptible (S) adding the number of plants with RB3 - 3 and TAN - 4 lesions.

Linkage map analysis was performed to each mapping population (10 populations) using the MSTmap software (http://mstmap.org/mstmap_online.html), with *Single LG* to grouping LOD (logarithm of the odds), a threshold of 15 cM to no mapping distance, and Kosambi mapping function to convert recombination values into map distances (cM). Linkage maps were constructed targeting regions (Table 2) associated with *Rpp* genes (Fig. 4, Supplementals Fig. S1 and Fig. S2).

QTL mapping was performed using the composite interval mapping (CIM) functionality in the R package qtl (Broman et al. 2003). QTL positions for lesion type were defined as the peaks of maximum LOD score, and the significance thresholds were calculated by a 1000 permutation test analysis at $\alpha \leq 0.05$ significance level. QTL intervals were estimated via loading function, using 1.5-LOD support confident intervals. Additive allelic effects were estimated by substituting resistant allele (AA) to susceptible allele (BB). Single marker regression analysis was performed for each marker to test the significant association between markers and the ASR phenotypes and determine the phenotypic variation explained by each KASP marker

RESULTS AND DISCUSSION

Phenotype resistance

The susceptible parents 55I57RSF IPRO and 63I64RSF IPRO produced TAN lesions, confirming the pathogen presence and susceptibility (Fig. 1, Fig. 2 and Fig. 3). Differences were observed in phenotypic response to ASR among *Rpp1* sources (Fig. 1 and Fig. 2). PI 200492 (*Rpp1*, Chr 18) showed high susceptibility (RB3 lesions), and immune (IM) response was observed by PI 594538A (*Rpp1-b*, Chr 18) and PI 587880A (*Rpp1-b*, Chr 18). PI 594723 (*Rpp1**) carrying unknown *Rpp1* allele, previously unmapped, showed strong resistance to ASR, with RB1 lesion type.

Population 1 (P1 – *Rpp1*) did not fit the expected segregation ratio of 3:1 (Table 3). P2 – *Rpp1-b* and P3 – Rpp1-b, with the Rpp1 allele variation in resistance sources PI 594538A (Rpp1-b) and PI 587880A (Rpp1-b), respectively, fit the expected phenotypic segregation (3:1), showing immunity (IM) reaction to ASR (Table 3 and Fig. 2). In P2 – *Rpp1-b*, 28% of the 75 plants showed an immune response to *P. pachyrhizi*, and in P3, 23% of 70 plants presented immune response (Fig. 2b and 2c). PI 594723 (*Rpp1**) revealed strong resistance with reddish brown lesions and no visible sporulation (RB1). P4 – *Rpp1** and P5 – *Rpp1** fit the expected segregation ratio (Table 3). With a total of 69 and 298 plants, these populations showed 50.7% and 68.5% of the plants as RB1 and RB2, respectively (Fig. 2d, Fig. 3a).

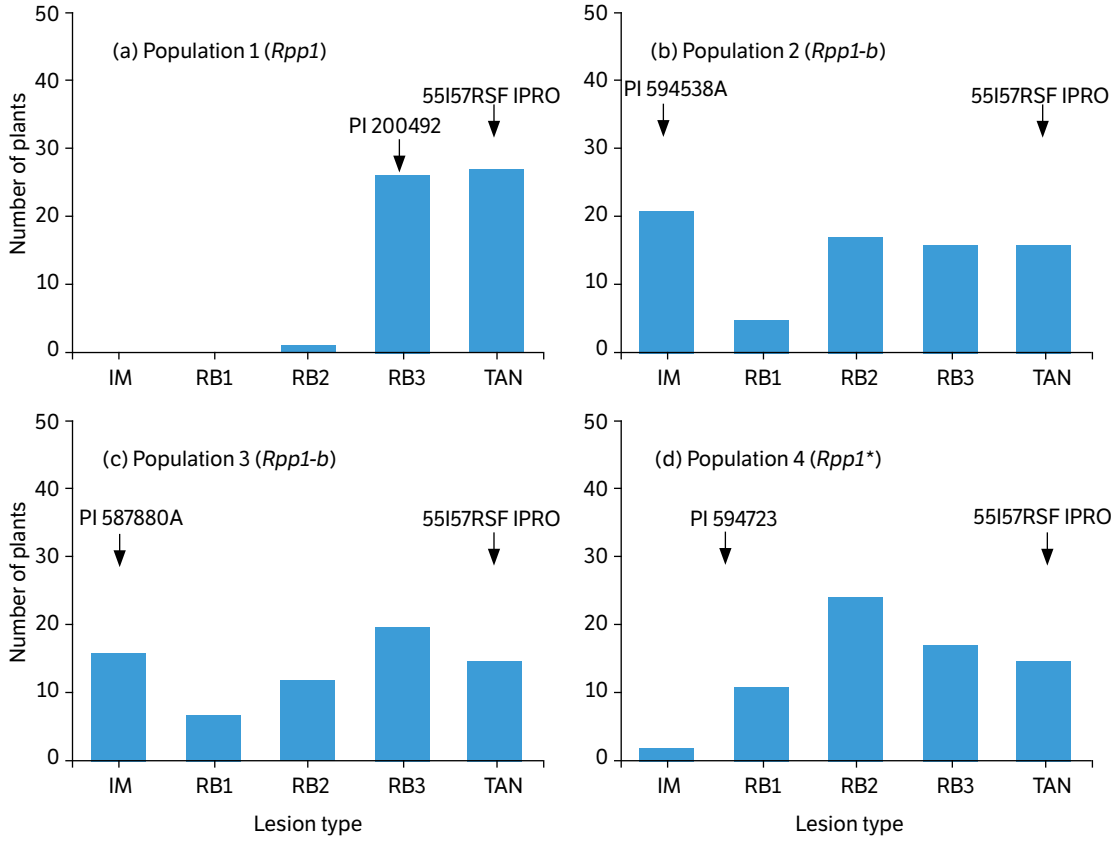

Figure 2. Frequency distributions of phenotypic reactions to Asian soybean rust (ASR) in F_2 soybean populations performed the 2017/18 crop season. a) Population 1 - *Rpp1* (55I57RSF IPRO x PI 200492. b) Population 2 - *Rpp1*-b (55I57RSF IPRO x PI 594538A. c) Population 3 - *Rpp1*-b (55I57RSF IPRO x PI 587880A). d) Population 4 - *Rpp1** (55I57RSF IPRO x PI 594723).

Source: Elaborated by the authors.

The resistant parent of P6 – *Rpp2* (PI 230970, Chr 16) showed a strong phenotypic reaction as RB1 lesions. P6 – *Rpp2* with 277 plants presented 42% of the plants with RB2 lesions, and 17% and 32% of the plants showed RB1 and RB3 phenotypic reactions to ASR, respectively (Fig. 3b). PI 506764 carries *Rpp3* (Chr 6) and *Rpp5* (Chr 3) genes and presented an RB2 reaction to ASR (Fig. 3c). P7 - *Rpp3*, 5 has PI 506764 resistant genes and showed a weak resistance to ASR, with over 70% of 288 plants presenting RB2 or RB3 lesions.

Population P8 - *Rpp4* showed weak resistance to ASR with 79% of the plants with RB2 or RB3 lesions of 291 plants. This weak resistance to ASR is related to the disease reaction from the resistance source PI 459025A (Fig. 3d). P9 - *Rpp5* and P10 - *Rpp5*, with 284 plants each population, carried the resistant allele *Rpp5* (Chr 3) from PI 200487 and showed no sporulation lesion type (RB1) in only 14% of the plants, RB2 in ~40%, and RB3 in 25% to 29.6% (Fig. 3e and 3f).

Mapping of resistance loci to ASR

Genotypic data revealed an association between the phenotypic reaction to ASR and KASP markers for all populations evaluated, except for P1 – *Rpp1* (PI 200492) (Table 4 and Supplemental Table S3). In P1, no markers showed significant association to phenotypic reaction (Supplemental Table S3). P2 and P3, carrying *Rpp1-b* (Chr 18) of PI 594538A and PI 587880A, respectively, presented a QTL in the same region, with a LOD peak at marker M6, and an additive effect ranged from 1.70 to 1.77 (Table 4). The QTL identified in P2 and P3 was responsible for 68 and 57% of the phenotypic reaction to ASR. The resistance locus *Rpp1-b* in P2 was mapped between M5 and M10 (6.5 cM) and between M4 and M10 (8.2 cM) in P3 (Supplemental Figure S1). The Chi-square (x^2) test revealed that all KASP markers mapped in P2 and P3 satisfactorily fitted the expected ratio for co-dominant inheritance (1:2:1) (Supplemental Table S3).

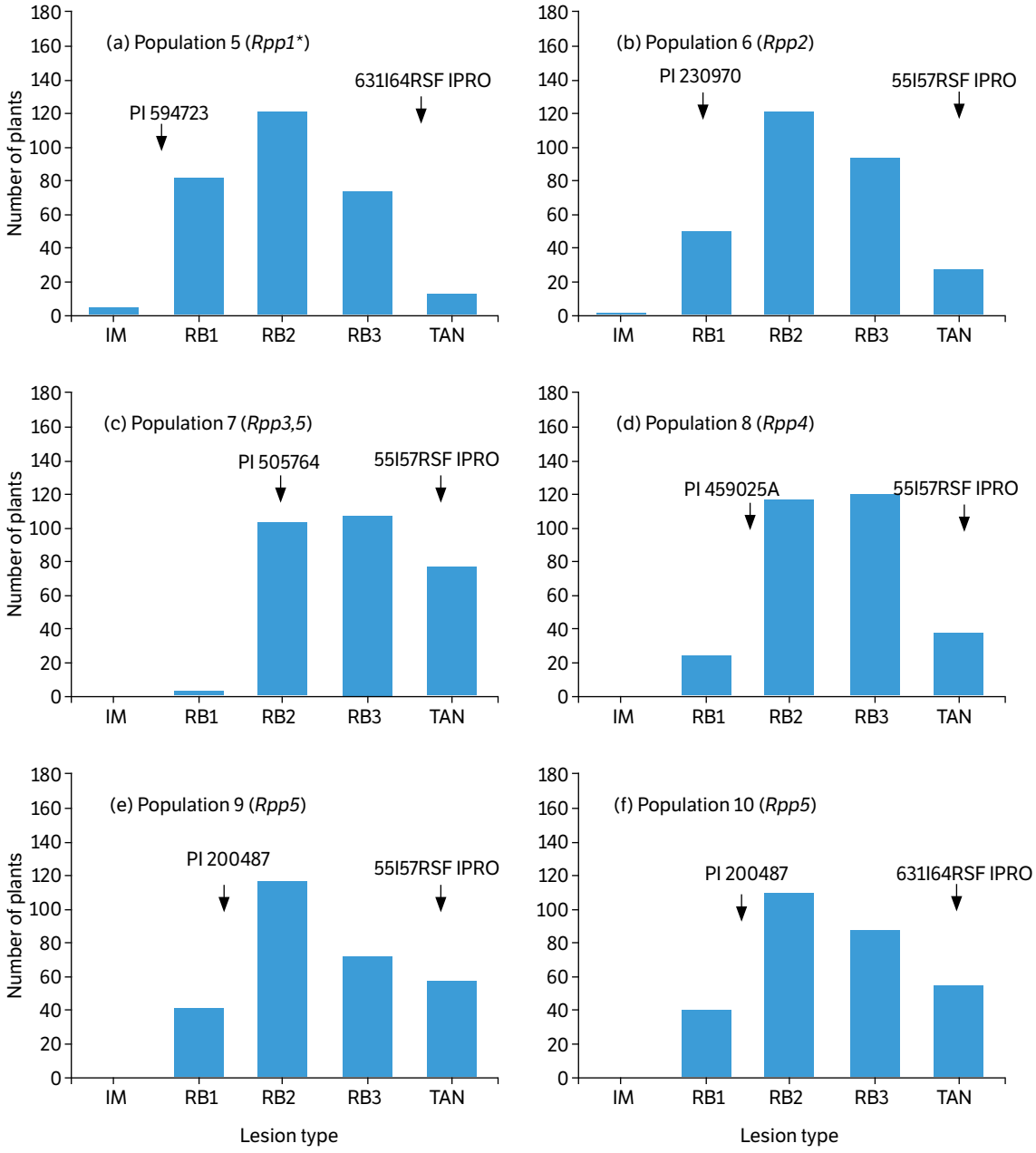

Figure 3. Frequency distributions of phenotypic reaction to Asian soybean rust (ASR) in F_2 soybean populations performed in 2018/2019 crop season. a) Population 5 - *Rpp1** (63I64RSF IPRO x PI 594723); b) Population 6 – *Rpp2* (55I57RSF IPRO x PI 230970); c) Population 7 – *Rpp3*, 5 (55I57RSF IPRO x PI 506764); d) Population 8 – *Rpp4* (55I57RSF IPRO x PI 459025A); e) Population 9 – *Rpp5* (55I57RSF IPRO x PI 200487); f) Population 10 – *Rpp5* (63I64RSF IPRO x PI 200487).

Source: Elaborated by the authors.

PI 594723 presented strong resistance to ASR (Fig. 2d and Fig. 3a), and it was hypothesized that PI 594723 carries an unknown *Rpp1** gene. A significant QTL was detected in P4 – *Rpp1** (PI 594723) between markers M1 and M6 on Chr 18 (Table 4) and validated in a different genetic background (P5). The QTL on P4 and P5 accounted for 42.2 and 27.8% of the phenotypic variation (Table 4 and Fig. 4). The additive effects of this locus to increase susceptibility ranged from 0.67 to 0.80. In P6 – *Rpp2* (PI 230970), a QTL was identified on Chr 16 between markers M13 and M14 (3 cM), with the peak at M14 and explaining 14.1% of the phenotypic variation for ASR resistance in the population (Table 4, Supplemental Fig. S1). All markers were significantly associated with ASR (Supplemental Table S3).

Table 4. Summary of quantitative trait loci (QTL) for lesion type to Asian soybean rust (ASR) in ten F_2 soybean populations.

Pop[a]	Gene	PI source	Chr.[b]	Position (cM) of LOD peak	Flanking Marker	Marker interval		1.5 interval (cM)	LOD[d]	P[e]	R^{2} [f]	Additive effect[g]	Dominance effect[g]
1	*Rpp1*	PI 200492	18	-c	-	-	-	-	-	-	-	-	-
2	*Rpp1-b*	PI 594538A	18	11.0	M6	M10	M5	7.3 - 13.8	22.0	<0.001	68.3	1.77	0.40
3	*Rpp1-b*	PI 587880A	18	3.0	M6	M10	M4	0.0 - 8.2	15.8	<0.001	57.6	1.70	0.54
4	*Rpp1**	PI 594723	18	7.0	M5	M6	M1	2.7 - 12.4	9.7	<0.001	42.2	0.80	0.48
5	*Rpp1**	PI 594723	18	2.1	M6	M6	M1	2.1 - 9.8	20.9	<0.001	27.8	0.67	0.24
6	*Rpp2*	PI 230970	16	1.0	M14	M14	M13	0.3 - 3.3	9.1	<0.001	14.1	0.42	0.35
7	*Rpp5*	PI 506764	3	0.0	M28	M28	M27	0.0 - 8.1	3.1	<0.001	5.3	0.15	-0.06
	Rpp3		6	13.0	M17	M20	M16	6.8 - 15.9	7.2	<0.001	12.4	0.37	-0.20
8	*Rpp4*	PI 459025A	18	3.0	M26	M26	M22	3.0 - 29.6	2.2	0.009	3.2	0.29	-0.14
9	*Rpp5*	PI 200487	3	0.0	M28	M27	M28	0.0 - 4.4	3.6	<0.001	5.8	0.44	0.08
10	*Rpp5*	PI 200487	3	0.7	M27	M27	M28	0.0 - 0.7	4.1	<0.001	6.4	0.38	0.26

[a] Population, crosses between the soybean rust-susceptible parental and resistant sources, described on Table 1. [b] Chr., Chromosome. [c] There were no LOD peaks above the threshold to population F2. [d] LOD, the logarithm of the odds. [e] P, probability of significance, calculated by single-factor analysis of variance. [f] R^2, coefficient of determination calculated based on the nearest marker by regression analysis. [g] Positive additive and dominance effects represent an increase in the value of the trait when the resistant allele (AA) is substituted with the susceptible allele (BB), and negative effects a decrease in the value of the trait. Source: Elaborated by the authors.

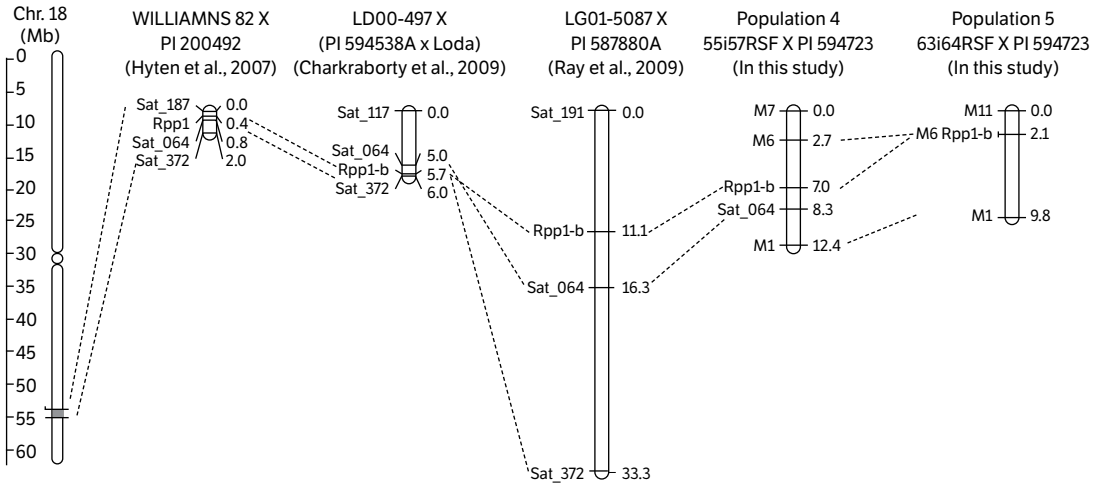

Figure 4. Compared linkage map location of *Rpp1* conferring resistance to Asian soybean rust (ASR) on Chr 18 with the location of Rpp1 mapped in PI 200492 by (Hyten et al. 2007), *Rpp1*-b mapped in PI 594538A by (Chakraborty et al. 2009), *Rpp1*-b mapped in PI 587880A by (Ray et al. 2009), and *Rpp1*-b mapped in this study in PI 594723. Map location of Rpp1-b in PI 594723 was based on the segregation of two trials composed of 69 and 298 F_2 soybean plants for Population 4 - *Rpp1** (55i57RSF x PI 594723) and Population 5 - *Rpp1** (63i64RSF x PI 594723).

Source: Elaborated by the authors.

The *Rpp3* and *Rpp5* loci were confirmed in population P7, and all KASP markers used were associated ($P \leq 0.002$) with ASR (Table 4). The *Rpp5* locus was mapped between markers M28 and M27 on Chr 3, and explained 5.8% of the phenotypic variation for ASR (Table 4). *Rpp3* was mapped on Chr 6 between markers M20 and M17 (Supplemental Fig. S2) and explained 12.4% of the phenotypic response to ASR resistance (Table 4).

Genotypic data revealed a QTL for ASR on Chr 18 between the markers M26 and M22 on P8 – *Rpp4* (Table 4 and Supplemental Fig. S2), confirming the *Rpp4* locus in the PI 459025A (Silva et al. 2008). M26 was associated ($P \leq 0.009$) with ASR and explained 3.17% of the phenotypic variation for the trait (Supplemental Table S3). Populations P9 and P10 have the *Rpp5* from PI 200487. The *Rpp5* locus was mapped between the markers M27 and M28 (Supplemental Fig. S2) and QTL explained 5.8% to 6.4% of the phenotypic variation (Table 4). The additive effect for the *Rpp* locus ranged from 0.38 to 0.44.

Several factors could lead to inconsistent results in the segregation ratio of F_2 soybean populations evaluated in this study. The trials were conducted in field conditions, where the combination of different ASR isolates, natural infection, and weather conditions could promote high inoculum pressure. In addition, the ASR isolates presented in Brazil are considered more virulent than ones found in Japan, Argentina, and Paraguay (Aoyagi et al. 2020; Yamanaka et al. 2010.

A comparison of ASR reactions of PI 594723 with other PIs carrying *Rpp1* (PI 200492) and *Rpp1-b* (PI 594538A and PI 587880A) showed higher similarity to *Rpp1-b* phenotypic response. The PI 594723 presented strong resistance, while the PI 200492 (*Rpp1*) presented RB3 lesions, classified as weak resistance. The higher susceptibility against ASR from PI 200492 demonstrates the inefficiency in genetic control using *Rpp1* in the study conditions. Akamatsu et al. (2017) observed susceptibility response from PI 200492 against several South America (Brazil, Argentina, and Paraguay) rust isolates.

PI 587880A and PI 594538A were classified as immune to ASR, and showed less sporulation than PI 200492 (Fig. 2a). Aoyagi et al. (2020) reported clear differences in the ASR reactions from genotypes carrying *Rpp1* and *Rpp1-b* and among sources of *Rpp1* such as PI 587886, Himeshirazu, and PI 200492, showing infection reactions of susceptibility, high resistance, and immunity depending on the rust isolate. Panho et al. (2022) classified PI 587880A and PI 594538A (*Rpp1-b*) genotypes as resistant, while PI 200492 (*Rpp1*) was classified as susceptible to field isolates in Brazil. Our results suggested that *Rpp1-b* has a higher genetic control against ASR than *Rpp1*.

The phenotypic reaction against ASR and mapping location suggested that PI 594723 carries *Rpp1-b* locus. Ray et al. (2009) identified the gene *Rpp1-b* on Chr 18 from PI 587880A in the same region where we identified the *Rpp1** locus on P4 and P5. In a few studies performed with PI 594723, Miles et al. (2008) reported resistant RB lesion type, with reduced sporulation level and low severity, in greenhouse and field conditions in Paraguay. However, Li (2009) observed moderate resistance to Mississippi isolates.

Pedley et al. (2019) identified eight genes that encode leucine-rich repeat (NBS-LRR) protein at the *Rpp1* locus. Four of these genes contain a novel ubiquitin-like protease 1 (ULP1) domain (Pedley et al. 2019). Only three of these genes, R3 – R5, are located within markers Sct_187 and Sat_064 that define the *Rpp1* locus (Hyten et al. 2007). This might explain the immunity and hypersensibility reaction to ASR observed in this study to PI 587880A, PI 594538A, and PI 594723 carrying *Rpp1-b* (Fig. 1). Chakraborty et al. (2009) mapped *Rpp1-b* on PI 594538A between markers Sat_064 and Sat_372, which agree with the physical position of R6 to R8. The *Rpp1-b* mapped on PI 587880A was located between markers Sat_191 and Sat_187 (Ray et al. 2009), which is in the same physical position of R1 and R8 resistance genes. Thus, according to the mapping proposed of PI 594723, eight genes homologous to the NBS-LRR family of disease R genes could be present (Pedley et al. 2019).

The markers previously mapped to *Rpp1-b* in PI 594723 (M1, M6, and M11) were confirmed in the P5 - *Rpp1-b*. This population avoids pathogen infection through hypersensitive reactions, resulting in lesions without sporulation, known as RB1 (Fig. 1). This resistant source has great potential to be used in breeding for ASR resistance, especially in South America. The flanking and interval KASP marker used in these populations (P4 and P5 – *Rpp1-b*) allows it to select plants with strong resistance.

The PI 230970 carries the dominant gene *Rpp2* on Chr 16 (Hartwig and Bromfield, 1983; Silva et al. 2008). In addition, Yu et al. (2015) fine mapped *Rpp2* from PI 230970 into a 188.1 kb region. Our results confirmed the *Rpp2* gene from PI 230970 on Chr 16. However, the phenotypic segregation ratio for P6 – *Rpp2* did not follow the expected 3:1 ratio. An explanation for that may be the presence of multiple rust isolates in the area since we had a natural infestation. Garcia et al. (2008) observed a similar trend when they used a different ASR isolate than previously used by Bromfield and Hartwig (1980) to map *Rpp2* from PI 230970.

PI 506764 contains alleles of *Rpp3* and *Rpp5* and represents a natural case of gene pyramiding. *Rpp3* was mapped on Chr 6 between Satt307 and satt460 (Hyten et al. 2009), and *Rpp5* on Chr 3 between Sat_275 and Sat_280 (Kendrick et al. 2011). Aoyagi et al. (2020) genotyped soybean landraces (WV51 and WC61) carrying *Rpp3* and reported different phenotypic reactions to isolates, presenting slight resistance to Brazilian isolates agreeing with our results. The *Rpp5* (PI 200487), mapped in Chr 3 between markers Sat_275 and Sat_280 (Garcia et al. 2008) showed great potential to be used in breeding programs. Our results demonstrated the contribution of *Rpp5* to increase levels of resistance in pyramided lines containing *Rpp3* + *Rpp5* (Fig. S3).

The resistant source carrying *Rpp4* (PI 459025A) presented satisfactory resistance to ASR. According to Hossain and Yamanaka (2019), this PI showed strong resistance against 80% of isolates from Bangladesh and Japan. However, when submitted to South American isolates (Brazil, Argentina, and Paraguay), the PI 459025A showed resistance to 50% of the isolates. *Rpp4* and *Rpp1* were mapped in the same linkage group on Chr 18, and this region is considered a hotspot for ASR resistance in soybean (Hyten et al. 2007; Silva et al. 2008). Previous studies with *Rpp4* verified a biphasic response to ASR, proposing that the gene detect effectors in the haustoria developing stage due to one or more of the multiple TIR-NBS-LRR candidate genes in the region (Meyer et al. 2009). These authors support the hypothesis that susceptibility to ASR can be associated with small amino acid differences responsible for playing a key role in resistance.

Pyramiding resistant genes in a single line can confer more durable and broad-spectrum resistance to a pathogen. Yamanaka and Hossain (2019) observed highly resistance to ASR when combined in one line multiple *Rpp* genes depending on the isolate. The KASP markers validated in this study might be used in MAS strategies to pyramiding different *Rpp* genes in one single line.

CONCLUSION

In conclusion, based on a slight difference in map position and a different reaction to ASR of PI 200492, the data suggested that PI 594723 carries a resistance gene *Rpp1-b*. The PIs carrying *Rpp1-b* (PI 594723, PI 587880A, and 594538A) showed strong resistance to ASR and generated high resistance plants when crossed with susceptible commercial cultivars. A total of 26 KASP markers were significantly associated ($P < 0.01$) with ASR and successfully mapped the resistant loci *Rpp1, Rpp2, Rpp3, Rpp4*, and *Rpp5*. Among the 26 KASP markers M1, M5, and M6 (*Rpp1*), M13 and M14 (*Rpp2*), M16, M17 and M20 (*Rpp3*), M25 and M26 (*Rpp4*), and M27 and M28 (*Rpp5*) have the potential to be used in marker-assisted selection strategies.

AUTHORS' CONTRIBUTION

Conceptualization: Meira, D., Bez Batti, V. B.; **Methodology:** Meira, D., Bez Batti, V. B, Panho, M. C.; **Investigation:** Meira, D.; Panho, M. C., Bez Batti, V. B, Milioli, A. S., Barrionuevo, F., Bozi, A. H., Madella, L. A.; **Writing – original draft:** Meira, D.; Panho, M. C., Bez Batti, V. B, Woyann, L. G., Milioli, A. S., Beche, E., Madella, L. A.; **Writing – review & editing:** Meira, D., Woyann, L. G., Milioli, A. S., Beche, E. and Finatto, T.; **Supervision:** Benin. G., Brito Júnior, S. L., Malone, G., Finatto, T.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on https://doi.org/10.5281/zenodo.8350064

FUNDING

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
https://doi.org/10.13039/501100002322
Finance code 001

ACKNOWLEDGMENTS

We thank GDM Seeds for providing funds and resources to support this project.

DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interest.

REFERENCES

Akamatsu, H., Yamanaka, N., Soares, R.M., Ivancovich, A.G., Lavilla, M.A., Bogado, A.N., Morel, G., Scholz, R., Yamaoka, Y. and Kato, M. (2017). Pathogenic Variation of South American *Phakopsora pachyrhizi* Populations Isolated from Soybeans from 2010 to 2015. Japan Agricultural Research Quarterly, 51, 221–232. https://doi.org/10.6090/jarq.51.221

Aoyagi, L.N., Muraki, Y. and Yamanaka, N. (2020). Characterization of three soybean landraces resistant to Asian soybean rust disease. Molecular Breeding, 40, 53. https://doi.org/10.1007/s11032-020-01132-w

Boller, T. and Felix, G. (2009). A Renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology, 60, 379–406. https://doi.org/10.1146/annurev.arplant.57.032905.10534

Broman, K.W., Wu, H., Sen, Ś. and Churchill, G.A. (2003). R/qtl: QTL mapping in experimental crosses. Bioinformatics, 19, 889–890. https://doi.org/10.1093/bioinformatics/btg112

Bromfield, K.R. and Hartwig, E.E. (1980). Resistance to soybean rust and mode of inheritance. Crop Science, 20, 254–255.

Chakraborty, N., Curley, J., Frederick, R.D., Hyten, D.L., Nelson, R.L., Hartman, G.L. and Diers, B.W. (2009). Mapping and Confirmation of a New Allele at *Rpp1* from Soybean PI 594538A Conferring RB Lesion–Type Resistance to Soybean Rust. Crop Science, 49, 783–790. https://doi.org/10.2135/cropsci2008.06.0335

Chen, H., Zhao, S., Yang, Z., Sha, A., Wan, Q., Zhang, C., Chen, L., Yuan, S., Qiu, D., Chen, S., Shan, Z. and Zhou, X. (2015). Genetic analysis and molecular mapping of resistance gene to *Phakopsora pachyrhizi* in soybean germplasm SX6907. Theoretical Applied Genetic, 128, 733–743. https://doi.org/10.1007/s00122-015-2468-2

Childs, S.P., King, Z.R., Walker, D.R., Harris, D.K., Pedley, K.F.., Buck, JW., Boerma, H.R. and Li, Z. (2018). Discovery of a seventh *Rpp* soybean rust resistance locus in soybean accession PI 605823. Theoretical Applied Genetic 131, 27–41. https://doi.org/10.1007/s00122-017-2983-4

Fehr, W.R., Caviness, C.E., Burmood, D.T. and Pennington, J.S. (1971). Stage of Development Descriptions for Soybeans, *Glycine Max* (L.) Merrill1. Crop Science, 11, 929-931. https://doi.org/10.2135/cropsci1971.0011183X001100060051x

Garcia, A., Calvo, É.S., Souza K.R.A., Harada, A., Hiromoto, D.M. and Vieira, L.G.E. (2008). Molecular mapping of soybean rust (*Phakopsora pachyrhizi*) resistance genes: discovery of a novel locus and alleles. Theoretical Applied Genetic, 117, 545–553. https://doi.org/10.1007/s00122-008-0798-z

Godoy, C.V., Seixas, C.D.S., Soares, R.M., Marcelino-Guimarães, F.C., Meyer, M.C. and Costamilan, L.M. (2016). Asian soybean rust in Brazil: past, present, and future. Pesquisa agropecuária brasileira, 51, 407–421. https://doi.org/10.1590/S0100-204X2016000500002

Goellner, K., Loehrer, M., Langenbach, C., Conrath, U., Koch, E. and Schaffrath, U. (2010). *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. Molecular Plant Pathology, 11, 169–177. https://doi.org/10.1111/j.1364-3703.2009.00589.x

Hartwig, E.E. (1986). Identification of a Fourth Major Gene Conferring Resistance to Soybean Rust. Crop Science, 26. https://doi.org/10.2135/cropsci1986.0011183X002600060010x

Hartwig, E.E. and Bromfield, K.R. (1983). Relationships Among Three Genes Conferring Specific Resistance to Rust in Soybeans. Crop Science, 23. https://doi.org/10.2135/cropsci1983.0011183X002300020012x

Hossain, M.D.M. and Yamanaka, N. (2019). Pathogenic variation of Asian soybean rust pathogen in Bangladesh. Journal Genetic Plant Pathology, 85, 90–100. https://doi.org/10.1007/s10327-018-0825-0

Hossain, M.M., Akamatsu, H., Morishita, M., Mori, T., Yamaoka, Y., Suenaga, K., Soares, R.M., Bogado, A.N., Ivancovich, A.J.G. and Yamanaka, N. (2015). Molecular mapping of Asian soybean rust resistance in soybean landraces PI 594767A, PI 587905 and PI 416764. Plant Pathology, 64, 147–156. https://doi.org/10.1111/ppa.12226

Hyten, D.L., Hartman, G.L., Nelson, R.L., Frederick, R.D., Concibido, V.C., Narvel, J.M. and Cregan, P.B. (2007). Map Location of the *Rpp1* Locus That Confers Resistance to Soybean Rust in Soybean. Crop Science, 47, 837–838. https://doi.org/10.2135/cropsci2006.07.0484

Hyten, D.L., Smith, J.R., Frederick, R.D., Tucker, M.L., Song, Q. and Cregan, P.B. (2009). Bulked Segregant Analysis Using the GoldenGate Assay to Locate the Rpp3 Locus that Confers Resistance to Soybean Rust in Soybean. Crop Science, 49, 265–271. https://doi.org/10.2135/cropsci2008.08.0511

Kendrick, M.D., Harris, D.K., Ha, B.K., Hyten, D.L., Cregan, P.B., Frederick, R.D., Boerma, H.R. and Pedley, K.F. (2011). Identification of a Second Asian Soybean Rust Resistance Gene in Hyuuga Soybean. Phytopathology, 101, 535–543. https://doi.org/10.1094/PHYTO-09-10-0257

Langenbach, C., Campe, R., Beyer, S.F., Mueller, A.N. and Conrath, U. (2016). Fighting Asian Soybean Rust. Frontiers in Plant Science, 7.

Li, S. (2009). Reaction of Soybean Rust-Resistant Lines Identified in Paraguay to Mississippi Isolates of *Phakopsora pachyrhizi*. Crop Science, 49, 887–894. https://doi.org/10.2135/cropsci2008.06.0305

Li, S., Smith, J.R., Ray, J.D. and Frederick, R.D. (2012). Identification of a new soybean rust resistance gene in PI 567102B. Theoretical Applied Genetic, 125, 133–142. https://doi.org/10.1007/s00122-012-1821-y

Meira, D., Woyann, L.G., Bozi, A.H., Milioli, A.S., Beche, E., Panho, M.C., Madella, L.A., Barrionuevo, F., Marchioro, V.S. and Benin, G. (2020). Asian soybean rust: a scientometric approach of *Phakopsora pachyrhizi* studies. Euphytica, 216, 133. https://doi.org/10.1007/s10681-020-02667-x

Meyer, J.D.F., Silva, D.C.G., Yang, C., Pedley, K.F., Zhang, C., van de Mortel, M., Hill, J.H., Shoemaker, R.C., Abdelnoor, R.V., Whitham, S.A. and Graham, M.A. (2009). Identification and Analyses of Candidate Genes for *Rpp4*-Mediated Resistance to Asian Soybean Rust in Soybean. Plant Physiology, 150, 295–307. https://doi.org/10.1104/pp.108.134551

Miles, M.R., Bonde, M.R., Nester, S.E., Berner, D.K., Frederick, R.D. and Hartman, G.L. (2011). Characterizing Resistance to *Phakopsora pachyrhizi* in Soybean. Plant Disease, 95, 577–581. https://doi.org/10.1094/PDIS-06-10-0450

Miles, M.R., Morel, W., Ray, J.D., Smith, J.R., Frederick, R.D. and Hartman, G.L. (2008). Adult Plant Evaluation of Soybean Accessions for Resistance to *Phakopsora pachyrhizi* in the Field and Greenhouse in Paraguay. Plant Disease, 92, 96–105. https://doi.org/10.1094/PDIS-92-1-0096

Panho, M.C., Fernandes, R.A.T., Menegazzi, C.P., Campagnolli, O.R., Quadra, F.C., Madella, L.A., Meira, D., Malone, G., Brito Junior, S.L. and Benin, G. (2022). *Rpp*-Gene pyramiding confers higher resistance level to Asian soybean rust. Euphytica, 218, 172. https://doi.org/10.1007/s10681-022-03123-8

Pedley, K.F., Pandey, A.K., Ruck, A., Lincoln, L.M., Whitham, S.A. and Graham, M.A. (2019). *Rpp1* encodes a ULP1-NBS-LRR protein that controls immunity to *Phakopsora pachyrhizi* in soybean. MPMI 32, 120–133. https://doi.org/10.1094/MPMI-07-18-0198-FI

Ray, J.D., Morel, W., Smith, J.R., Frederick, R.D. and Miles, M.R. (2009). Genetics and mapping of adult plant rust resistance in soybean PI 587886 and PI 587880A. Theoretical Applied Genetic, 119, 271–280. https://doi.org/10.1007/s00122-009-1036-z

Silva, D.C.G., Yamanaka, N., Brogin, R.L., Arias, C.A.A., Nepomuceno, A.L., Di Mauro, A.O., Pereira, S.S., Nogueira, L.M., Passianotto, A.L., Abdelnoor, R.V. (2008). Molecular mapping of two loci that confer resistance to Asian rust in soybean. Theoretical Applied Genetic, 117, 57–63. https://doi.org/10.1007/s00122-008-0752-0

Yamanaka, N. and Hossain, M.D.M. (2019). Pyramiding three rust-resistance genes confers a high level of resistance in soybean (*Glycine max*). Plant Breeding, 138, 686–695. https://doi.org/10.1111/pbr.12720

Yamanaka, N., Morishita, M., Mori, T., Muraki, Y., Hasegawa, M., Hossain, M.D.M., Yamaoka, Y., and Kato, M. (2016). The locus for resistance to Asian soybean rust in PI 587855. Plant Breeding, 135, 621–626. https://doi.org/10.1111/pbr.12392

Yamanaka, N., Yamaoka, Y., Kato, M., Lemos, N.G., Passianotto, A.L.L., Santos, J.V.M., Benitez, E.R., Abdelnoor, R.V., Soares, R.M. and Suenaga, K. (2010). Development of classification criteria for resistance to soybean rust and differences in virulence among Japanese and Brazilian rust populations. Tropical plant pathology. 35, 153–162. https://doi.org/10.1590/S1982-56762010000300003

Yu, N., Kim, M., King, Z.R., Harris, D.K., Buck, J.W., Li, Z. and Diers, B.W. (2015). Fine mapping of the Asian soybean rust resistance gene *Rpp2* from soybean PI 230970. Theoretical Applied Genetic, 128, 387–396. https://doi.org/10.1007/s00122-014-2438-0